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<p>(54) Title: CONVERSION OF COMPACTIN TO PRAVASTATIN BY ACTINOMADURA (57) Abstract A method for converting compactin to pravastatin is described. Compactin is provided and contacted with an agent derived from Actinomadura under conditions in which the agent converts compactin to pravastatin. Also described are an Actinomadura strain, an Actinomadura cell free extract, an Actinomadura hydroxylase, and a method for lowering cholesterol levels in mammals.</p>		

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CONVERSION OF COMPACTIN TO PRAVASTATIN BY ACTINOMADURA

FIELD OF THE INVENTION

This invention relates to methods for converting compactin to pravastatin using an agent derived from the filamentous bacterium *Actinomadura*, to methods for lowering cholesterol levels in mammals, and to *Actinomadura* and to *Actinomadura* hydroxylase.

BACKGROUND OF THE INVENTION

One of the major causes of atherosclerosis and coronary disease is attributed to high blood cholesterol levels. It has been estimated that at least about 50% of total body cholesterol is derived from de novo cholesterol synthesis. A major rate-limiting step in the cholesterol biosynthetic pathway is catalyzed by 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase. Compactin and pravastatin have been reported to be competitive inhibitors of HMG-CoA reductase, and the presence of either one can result in inhibition of cholesterol biosynthesis.

Microbial hydroxylation of compactin can produce hydroxylated forms of compactin, e.g., pravastatin. Some hydroxylated forms are reportedly more effective than compactin as competitive inhibitors of HMG-CoA. It has been reported that this hydroxylation can be effected to differing degrees by many different genera of fungi, and from the bacteria *Nocardia* and *Streptomyces roseochromogenus* and *Streptomyces carbophilus*. See, e.g., U.S. Patent No. 5,179,013; U.S. Patent 4,448,979; U.S. Patent No. 4,346,227; U.S. Patent No. 4,537,859; Canadian Patent No. 1,150,170; Canadian Patent No. 1,186,647; Serizawa et al., *J. Antibiotics* 36:887-891 (1983).

A problem with using fungi for the production of pravastatin is that they generally do not tolerate increases in the amount of compactin added to the culture medium, presumably due to the anti-fungal activity of compactin. Serizawa et al., *J. Antibiotics* 36:887-891 (1983).

The cytochrome P450 system has been shown to be required for the hydroxylation of compactin to pravastatin in *Streptomyces carbophilus*. Matsuoka et al., *Eur. J. Biochem.* 184:707-713 (1989). Problems with the use of such an enzyme is that it is a complex of proteins rather than a single protein, making recombinant DNA manipulations difficult, and that compactin, which is an inducer of the cytochrome P450 system, is very expensive.

SUMMARY OF THE INVENTION

It is an object of the invention to provide an effective and relatively inexpensive method for converting compactin to pravastatin.

It is another object of the invention to use Actinomadura, a filamentous bacterium, to
5 convert compactin to pravastatin.

It is yet another object of the invention to use an Actinomadura hydroxylase to convert compactin to pravastatin.

It is yet another object of the invention to use an Actinomadura constitutive hydroxylase that does not require compactin as an inducer, to convert compactin to
10 pravastatin.

Still another object of the invention is to use pravastatin, derived from an Actinomadura hydroxylase that converts compactin to pravastatin, to treat a mammal so as to lower the mammal's blood cholesterol level.

According to the invention, a method for converting compactin to pravastatin is
15 provided. Compactin is provided and contacted with an agent, e.g., a hydroxylation enzyme, e.g., a hydroxylase that is, e.g., constitutive and cytochrome P450 system-independent, derived from Actinomadura, under conditions in which the agent converts compactin to pravastatin. In certain embodiments, the pravastatin is isolated.

Preferably, the compactin is provided by providing, e.g., a microorganism, e.g., a
20 fungus or bacterium, that produces compactin, or a cell free extract of a microorganism that produces compactin, or cell free culture media from a pregrown culture of a microorganism that produces compactin, or a solution comprising compactin, or semi-purified compactin, or substantially purified compactin.

In certain embodiments, the compactin is contacted with the agent, e.g., by contacting
25 whole cells of Actinomadura with the compactin, or by contacting a cell free extract of Actinomadura with the compactin, or by contacting cell free culture media from a pregrown culture of Actinomadura with the compactin, or by contacting a solution having the Actinomadura agent with the compactin, or by contacting semi-purified or substantially purified Actinomadura agent with the compactin.

30 Variations include, e.g., contacting, e.g., a culture, e.g., a pregrown culture or a starting culture, or a cell free extract, of the microorganism that produces compactin, or semi-purified or substantially purified compactin, with, e.g., a culture, e.g., a pregrown culture or a

starting culture, or a cell free extract, of Actinomadura, or semi-purified or substantially purified Actinomadura agent.

Another aspect of the invention is a cell free extract derived from Actinomadura, having an agent, e.g., a hydroxylase, that converts compactin to pravastatin.

5 Another aspect of the invention is a hydroxylase, e.g., semi-purified or substantially purified, from Actinomadura that converts compactin to pravastatin, wherein the hydroxylase is a constitutive enzyme, wherein the activity of the hydroxylase is stimulated by any of ATP, ascorbic acid or Mg^{++} , but not by Fe^{++} or Fe^{+++} , and wherein the hydroxylase is cytochrome P450 system-independent.

10 Another aspect of the invention is purified Actinomadura ATCC 55678 having an agent for converting compactin to pravastatin.

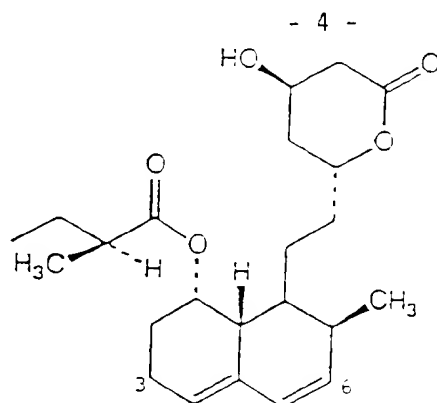
Yet another aspect of the invention is a method for treating a mammal to lower the blood cholesterol level of the mammal. Pravastatin, derived from compactin by contacting the compactin with an agent, e.g., a hydroxylase, derived from Actinomadura that converts
15 compactin to pravastatin, is provided. The pravastatin is administered to a mammal in need of such treatment to cause a lower blood cholesterol level in the mammal.

The above and other objects, features and advantages of the present invention will be better understood from the following specification.

20 DETAILED DESCRIPTION

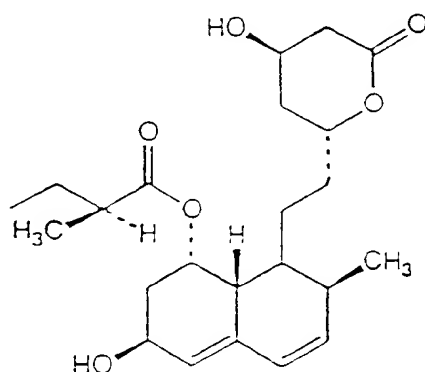
This invention provides a method for converting compactin to pravastatin. Compactin is provided and contacted with an agent derived from Actinomadura under conditions in which the agent converts compactin to pravastatin. In certain embodiments, the pravastatin is isolated.

25 Compactin (also known as mevastatin, ML-236B, and CS-500) is meant to include, e.g., the acid form (also known as ML-236B carboxylic acid), the lactone form (also known as ML-236B lactone), and salts and esters thereof. The lactone form of compactin may be represented by the formula (I):



A preferred compactin is the sodium salt of compactin.

Pravastatin (also known as eptastatin, mevalotin, pravachol, CS-514, and SQ-31000) is meant to include, e.g., the acid form, the lactone form, and salts and esters thereof. The 3 β -hydroxy lactone form of pravastatin may be represented by the formula (II):



Other forms of hydroxylated compactin include compounds in which the hydroxyl group is added at other positions of the compactin molecule, e.g., at position 6.

The compactin can be provided in any way which enables the agent to act upon it. For example, compactin can be provided by providing a microorganism, e.g., a fungus or bacterium, that produces compactin. Microorganism is meant to include, e.g., microbial cells which are intact, immobilized or permeabilized.

Production of compactin by the microorganism is meant to include the microorganism using its own natural gene or genes, or fragments thereof, to produce the compactin, and/or the

microorganism using a foreign gene or genes, or fragments thereof, to produce the compactin. The foreign gene can be introduced into the microorganism by standard molecular cloning techniques known in the art, or by any other means which will result in expression of the compactin producing gene or genes, or fragments thereof, in the microorganism. In certain
5 embodiments, the compactin is provided by using a cell free extract of a microorganism that produces compactin. The cell free extract can be prepared by a variety of methods known in the art, e.g., by physical or chemical means, so as to rupture the cells. Such methods include, e.g., grinding, ultrasonic treatment, or treatment with an enzyme or surface active agent. Preferably, the cell free extract is prepared with a French Press. Cell free extract is meant to
10 include the material resulting from any of the preparation methods, or the soluble fraction resulting from any of the preparation methods. Preferably, the cell free extract comprises essentially the cellular contents without the particulate cellular debris, e.g., walls, e.g., wherein the walls have been essentially removed. Cell free extracts can be made from cells obtained before, during or after active growth of the cells. In other embodiments, the
15 compactin can be provided by providing cell free culture media from a pregrown culture of a microorganism that produces compactin. In other embodiments, the compactin can be provided by providing a solution comprising compactin. In yet other embodiments, the compactin can be provided by using semi-purified or substantially purified compactin. The compactin can be free or immobilized. Any other source of compactin can be used in this
20 invention. Compactin can be used in this invention at any concentration which will result in production of pravastatin. Preferably, the compactin concentration is between about 0.1 and about 100 g/liter, more preferably is between about 0.2 and about 25 g/liter, and most preferably is between about 1 and about 10 g/liter. In some embodiments, the compactin is added to the Actinomadura agent in shots. By shots is meant sequential additions of the
25 agent. See Example 3.

The compactin is contacted with an agent derived from Actinomadura. Actinomadura is a genus belonging to the filamentous bacteria, actinomycetes. Actinomadura is meant to include wild type or any mutants which possess the ability to convert compactin to pravastatin. The mutants can be derived, e.g., spontaneously, or from physical agents, e.g.,
30 ultraviolet radiation, high frequency electromagnetic waves or nuclear radiation, or chemical agents, or from genetic engineering techniques.

Morphological and chemotaxonomic properties of a newly isolated strain included in this invention were compared with those of similar taxa and the results showed that the strain belongs to the genus *Actinomadura*. Deposit of these *Actinomadura* cells (strain 2966) has been made on June 1, 1995, with the American Type Culture Collection, 12301 Parklawn Drive, Rockville, MD 20852, and has been assigned deposit number ATCC 55678.

The agent derived from *Actinomadura* is meant to include, e.g., an enzyme, e.g., a hydroxylation enzyme, e.g., a hydroxylase, or any active portion thereof, that is able to convert compactin to pravastatin. The *Actinomadura* agent can be made in vivo or in vitro, e.g., in cell-free systems or by chemical synthesis. The agent includes wild type and mutated forms. The mutated agents can be derived spontaneously or from mutating the *Actinomadura*, e.g., with physical or chemical agents, or from utilizing genetic engineering techniques known to those skilled in the art. Mutated agents can also be synthesized by chemical techniques known to those skilled in the art. The agent is also meant to include, e.g., an agent derived from a genetically engineered strain of *Actinomadura* in which the nucleic acid coding for the agent or any active portion thereof, is operatively connected to a regulatory region, or portion thereof, that is different from the wild type regulatory region for the agent. In certain embodiments, the nucleic acid coding for the *Actinomadura* agent is cloned into another type of cell, e.g., a bacterium, e.g., *Escherichia coli*, *Bacillus subtilis*, *Bacillus brevis* or *Streptomyces lividans*; a fungus, e.g., *Saccharomyces cerevisiae*, *Pichia pastoris*, *Hansenula polymorpha*; insect cells; transgenic plants; or transgenic animals. In a preferred embodiment, the *Actinomadura* agent is cloned into a microorganism which is able to produce compactin, e.g., with the microorganism's own gene(s), or with foreign gene(s).

The agent of this invention is a constitutive enzyme, and therefore does not require induction with compactin. See Example 6. Mg^{++} , ATP and ascorbic acid, alone or in combination, but not Fe^{++} or Fe^{+++} , stimulates the enzymatic activity of the agent. See Example 8. Preferably, NADPH is used as a H^+ donor, though NADH or other H^+ donors can be used. Cofactors, e.g., α -ketoglutarate, $CoCl_2$, $NiCl_2$, $CuSO_4$, FMN or FAD, do not stimulate enzymatic activity of the agent. The agent is cytochrome P450 system-independent. See Example 7.

The compactin can be contacted with the agent in any way which enables the agent to convert the compactin to pravastatin. For example, the compactin can be contacted with whole cells of *Actinomadura*, or with a cell free extract of *Actinomadura*, or with cell free

culture media from a pregrown culture of Actinomadura, or with a solution comprising the Actinomadura agent, or with semi-purified or substantially purified Actinomadura agent.

In certain embodiments, a pregrown culture of a microorganism that produces compactin is contacted with a pregrown culture of Actinomadura. By pregrown culture is meant a culture which has been inoculated with cells and cultured for some period of time, preferably about 1 to about 8 days, more preferably about 1 to about 5 days, and most preferably about 2 to about 4 days. In other embodiments, a pregrown culture of a microorganism is contacted with a starting culture of Actinomadura. By starting culture is meant a culture which is inoculated with cells from, e.g., a slant culture, a frozen culture, a lyophilized culture or a liquid seed culture. In other embodiments, a starting culture of a microorganism that produces compactin is contacted with a pregrown culture of Actinomadura. In other embodiments, a starting culture of a microorganism that produces compactin is contacted with a starting culture of Actinomadura. This mixture of cultures is grown for some period of time, preferably about 0.5 to about 8 days, more preferably about 1 to about 5 days, and most preferably about 2 to about 4 days.

In other embodiments, a cell free extract of a microorganism that produces compactin is contacted with a culture, e.g., starting or pregrown, of Actinomadura. In other embodiments, a culture, e.g., starting or pregrown, of a microorganism that produces compactin is contacted with a cell free extract of Actinomadura. And, in yet other embodiments, a cell free extract of a microorganism that produces compactin is contacted with a cell free extract of Actinomadura. In other embodiments, a culture, e.g., starting or pregrown, of a microorganism that produces compactin is contacted with a cell free extract of Actinomadura. Combinations using semi-purified or substantially purified compactin, or semi-purified or substantially purified Actinomadura agent are also included in this invention.

The conversion reaction of compactin to pravastatin can be done using any conditions which will result in the production of pravastatin. Any method of cultivation can be used, e.g., fermentation techniques, e.g., batch culture, fed-batch culture, continuous or solid-state culture. Preferably, an agitated liquid submerged culture is used, most preferably of the type useful for large scale industrial fermentation. Additives can be used during growth which contribute to the development of the hydroxylating system within the cells.

The preferred temperature is about 18°C to about 50°C, more preferably about 25°C to about 37°C, and most preferably about 28°C to about 31°C. The preferred pH is about 5 to

about 10, more preferably about 6 to about 8.5, and most preferably about 7.2 to about 8.0. Preferably, aerobiosis is provided, e.g., by agitation and/or aeration. The preferred shaking condition is about 0 rpm to about 400 rpm, more preferably about 200 rpm to about 250 rpm, and most preferably about 220 rpm.

5 The invention is meant to cover any percentage of conversion of compactin to pravastatin by the *Actinomadura* agent, preferably at least about 10%, more preferably at least about 25%, more preferably yet at least about 40%, more preferably yet at least about 50%, more preferably yet at least about 60%, more preferably yet at least about 70%, and most preferably at least about 80%. The percentage conversion is calculated by dividing the
10 concentration of pravastatin by the concentration of compactin charged (initially added) and multiplying by 100.

 In preferred embodiments, the pravastatin is isolated. Isolated is meant to include, e.g., enriched, separated or purified. Isolation can be by any method known to those skilled in the art, including, e.g., precipitation; extraction, e.g., with a solvent, e.g., ethyl acetate or
15 butanol, and removal of the solvent, e.g., by distillation; chromatography, e.g., thin layer chromatography or column chromatography, e.g., using a matrix, e.g., alumina or silica gel, followed by elution. A preferred chromatographic method includes, e.g., high pressure liquid chromatography (HPLC). See, e.g., Serizawa et al., *J. Antibiotics* (1983).

 The invention also includes a cell free extract from *Actinomadura* that has an agent,
20 e.g., a hydroxylase, that converts compactin to pravastatin. The cell free extract can be obtained by any method, including those described above. Preferably, the cell free extract comprises essentially the cellular contents without particulate cellular debris, e.g., walls, e.g., wherein the walls have been essentially removed. Preferably, conversion of compactin to pravastatin by the cell free extract is at least about 10%, more preferably at least about 25%,
25 more preferably yet at least about 40%, more preferably yet at least about 50%, more preferably yet at least about 60%, more preferably yet at least about 70%, and most preferably at least about 80%.

 The invention also includes a hydroxylase, e.g., semi-purified or substantially purified, from *Actinomadura* that converts compactin to pravastatin. The hydroxylase is a constitutive
30 enzyme. The activity of the hydroxylase is stimulated by any of ATP, ascorbic acid and Mg^{++} , or by combinations thereof, but not by Fe^{++} or Fe^{+++} . Preferably, NADPH is used as a H^+ donor, though NADH or other H^+ donors can be used. Cofactors, e.g., α -ketoglutarate,

CoCl₂, NiCl₂, CuSO₄, FMN or FAD, do not stimulate the activity of the hydroxylase. The hydroxylase is cytochrome P450 system-independent.

The invention also includes Actinomadura ATCC 55678, having an agent, e.g., a hydroxylase, for converting compactin to pravastatin.

5 The invention further includes a method for treating a mammal to lower the blood cholesterol level of the mammal. Pravastatin, derived from compactin by contacting the compactin with an agent, e.g., a hydroxylase, derived from Actinomadura that converts compactin to pravastatin, is provided. The pravastatin is administered to a mammal in need of such treatment to cause a lower blood cholesterol level in the mammal.

10 By mammal is meant human as well as non-human mammals. Treating a mammal to lower blood cholesterol levels is meant to include, e.g., preventing or lowering high blood cholesterol levels.

Administration of the pravastatin can be accomplished by any method which allows the pravastatin to reach its target. By target is meant the place where the pravastatin is able to
15 inhibit the cholesterol biosynthetic pathway in the mammal. The administration methods include, e.g., injection, deposition, implantation, suppositories, oral ingestion, inhalation, topical administration, or any other method of administration where access to the target by pravastatin is obtained. Injections can be, e.g., intravenous, intradermal, subcutaneous, intramuscular or intraperitoneal. Implantation includes inserting implantable drug delivery
20 systems, e.g., microspheres, hydrogels, polymeric reservoirs, cholesterol matrices, polymeric systems, e.g., matrix erosion and/or diffusion systems and non-polymeric systems, e.g., compressed, fused or partially fused pellets. Suppositories include glycerin suppositories. Oral ingestion doses, e.g., pills, can be enterically coated. Inhalation includes administering pravastatin with an aerosol in an inhalator, either alone or attached to a carrier that can be
25 absorbed.

The pravastatin can be suspended in a liquid, e.g., in dissolved form or colloidal form. The liquid can be a solvent, partial solvent or non-solvent. In many cases, water or an organic liquid can be used. Administration of pravastatin can be alone or in combination with other therapeutic agents. In certain embodiments, the pravastatin can be combined with a suitable
30 carrier, e.g., a pharmaceutically acceptable carrier. In certain embodiments, the pravastatin is incorporated into a liposome or incorporated into a polymer release system.

In certain embodiments of the invention, the administration can be designed so as to result in sequential exposures to the pravastatin over some time period, e.g., hours, days, weeks, months or years. This can be accomplished by repeated administrations of the pravastatin by one of the methods described above, or alternatively, by a controlled release
5 delivery system in which the pravastatin is delivered to the mammal over a prolonged period without repeated administrations. Administration of such a system can be, e.g., by long acting oral dosage forms, bolus injections, transdermal patches and sub-cutaneous implants.

The pravastatin can be administered prior to or subsequent to the appearance of high blood cholesterol levels. In certain embodiments, the pravastatin is administered to subjects
10 who have a family history of high blood cholesterol levels, or who have a genetic predisposition for this condition. In other embodiments, the pravastatin is administered to subjects who have reached a particular age and who, therefore, are more likely to be affected by high blood cholesterol levels. In yet other embodiments, the pravastatin is administered to subjects who exhibit either early or advanced symptoms of the condition. The pravastatin can
15 also be administered as a preventive measure.

The pravastatin is administered to the mammal in a therapeutically effective amount. By therapeutically effective amount is meant that amount which is capable of at least partially preventing or reversing high blood cholesterol levels. A therapeutically effective amount can be determined on an individual basis and will be based, at least in part, on consideration of the
20 species of mammal, the mammal's size, the type of delivery system used, the time of administration relative to the level of blood cholesterol, and whether a single, multiple, or controlled release dose regimen is employed. A therapeutically effective amount can be determined by one of ordinary skill in the art employing such factors and using no more than routine experimentation.

25 Preferably, the dosage of pravastatin for a human is about 0.1 to about 5000 mg/day, more preferably it is about 1 to about 500 mg/day, and most preferably it is about 10 to about 50 mg/day. Preferably, the dosage form is such that it does not substantially deleteriously affect the mammal.

EXAMPLES

Example 1: Bioconversion of Compactin to Pravastatin by Intact Cells of Actinomadura

This example illustrates the bioconversion of compactin to pravastatin by
5 Actinomadura cells. Actinomadura ATCC 55678 was grown in YM medium (yeast extract
3g, malt extract 3g, peptone 5g, glucose 10g, per liter, pH 6.5) for 48 hours, at 28°C. with
shaking at 220 rpm. 500 µg/ml Na compactin (obtained from Fluka Chemical Corp., New
York, NY) was then added. The Actinomadura cultures were further incubated in the same
medium for 1, 2, 3, 4 or 5 days. At the end of each of these time periods, the amount of
10 compactin (CP) and pravastatin (PV) was determined by high performance liquid
chromatography (HPLC).

The HPLC conditions used were a 3 µm Lichrospher 100 RP-18 column (obtained
from EM Separations Technology, Gibbstown, NJ); Mobile Phase A: 2.5g NaH₂PO₄ in 800
ml Milli-Q water, add 200 ml acetonitrile and 110 ml methanol, adjust pH to 2.5 with 85%
15 H₃PO₄; Mobile Phase B: mixture of 200 ml water, 800 ml acetonitrile, 110 ml methanol and
1 ml 85% H₃PO₄; a 10 µl injection volume; a 1 ml/min. flow rate; and detection at UV 257
nm. The gradient table was:

Time (min.)	A%	B%	Curve
20 0	90	10	-
5	90	10	11
35	20	80	6
40	90	10	6
55	90	10	11

25

The HPLC results are shown in Table 1. PV is pravastatin; CP is compactin.

Table 1. Bioconversion by Actinomadura During 5 Days Incubation

days	1	2	3	4	5
PV	252	262	290	318	326
CP	32	0	0	0	5.4
% conversion	50.4	52.4	58.0	63.6	65.2

unit: $\mu\text{g/ml}$

- 10 % conversion was calculated on the basis of compactin charged, not compactin used (the compactin was weighed before addition, but the charge was determined by HPLC assay at time zero).

15 Example 2: Kinetics of Bioconversion of Compactin to Pravastatin by Intact Cells of Actinomadura

This example illustrates the kinetics of the bioconversion of compactin to pravastatin by Actinomadura. A culture medium containing 2% glucose, 0.5% peptone, 0.3% yeast extract and 0.3% malt extract was prepared and adjusted to pH 5.5. Erlenmeyer flasks of 250 ml volume, each of which contained 20 ml of the medium, were sterilized at 121°C for 15 minutes. Each volume was then inoculated with a platinum loop containing cells of Actinomadura ATCC 55678 from a slant culture, and then cultured with shaking at 250 rpm, 28°C for 5 days. This procedure resulted in a suspension containing pellets plus turbid liquid. 2 ml of the turbid part of the culture was inoculated into another 250 ml Erlenmeyer flask with 20 ml medium and incubated shaking at 250 rpm, 28°C for 2 days, giving a seed culture. 50 ml aliquots of the above medium were added to 500 ml Erlenmeyer flasks, and sterilized at 121°C for 15 minutes. 5 ml of seed culture was added into each flask. After 2 days, compactin was added into each flask to a level of 500 $\mu\text{g/ml}$ and further cultured for 36 hours. The rate of pravastatin production at different time intervals during 36 hours of bioconversion was determined as described in Example 1. The results are shown in Table 2.

Table 2. Bioconversion by Actinomadura During
36 Hours Incubation

Time (hours)	0	4	8	12	17	20	24	30	33	36
PV	0	62.7	109	158	205	224	238	258	263	274
CP	502	390	301	209	111	79.2	84.9	55.3	46.2	6.9
% conversion	0	12.5	21.7	31.5	40.8	44.6	47.4	51.4	52.4	54.6

unit: $\mu\text{g/ml}$

For 16 hours both pravastatin production and compactin consumption were linear.

Additional experiments were conducted to examine bioconversion at shorter time intervals over 7 hours, at compactin concentrations of 200 $\mu\text{g/ml}$ and 500 $\mu\text{g/ml}$. The results are shown in Tables 3 and 4.

Table 3. 200 $\mu\text{g/ml}$ Compactin

Time (hours)	0	0.5	1	2	3	4	5	6	7
PV	0	3.5	6.5	12.7	18.8	24.5	28.8	33	48.7
CP	227	199	194	184	174	163	156	152	130
% conversion	0	1.5	2.9	5.6	8.3	10.8	12.7	14.5	21.5

unit: $\mu\text{g/ml}$

Table 4. 500 µg/ml Compactin

Time (hours)	0	0.5	1	2	3	4	5	6	7
PV	0	3.6	6.8	13.2	18.9	24.5	28.9	32.6	54.1
CP	494	493	488	477	466	459	455	452	430
% conversion	0	0.6	1.4	2.7	3.8	5.0	5.9	6.6	11

unit: µg/ml

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From the above results, it is clear that there was essentially no difference between the two compactin concentrations in the rate of pravastatin production.

Example 3: Bioconversion of Compactin to Pravastatin By Intact Cells of Actinomadura

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When the Compactin is Added in Shots

This example illustrates that the pravastatin concentration resulting from the bioconversion of compactin to pravastatin by intact cells of Actinomadura is increased if the compactin is added in shots. Actinomadura ATCC 55678 was grown as described in Example 2, with 500 µg/ml compactin added as described. An additional 300 µg/ml compactin was added at the first day, and yet another additional 300 µg/ml compactin was added at the second day. The amounts of compactin and pravastatin were measured as described in Example 1. The results are shown in Table 5.

20

Table 5. Bioconversion by Actinomadura With
Compactin Added in Shots

5	Time (days)	0	1	1 (after CP added)	2	2 (after CP added)	3	4	5	6	7
	PV	0.8	271	258	398	380	479	536	585	684	821
	CP	481	0	304	37.0	299	146	85.5	69.7	64.7	78.0
	total added CP	481		785		1047					
10	% conversion		57.3		50.7		46.0	51.2	55.9	65.3	78.4

unit: $\mu\text{g/ml}$

The results indicate that very high concentrations of pravastatin (821 $\mu\text{g/ml}$) were
 15 obtained after 7 days when the compactin was added in shots. Experiments conducted under
 similar conditions except for compactin being added only once (no shots), gave lower
 concentrations of produced pravastatin. In addition, as shown in Table 5, the percent
 bioconversion was increased to 78% in the shots experiment. Thus, adding compactin in
 shots led to higher pravastatin concentrations and higher percent conversion. The rate of
 20 bioconversion of the compactin to pravastatin was not increased.

Example 4: Growth and Bioconversion of Compactin to Pravastatin
by Cells of Actinomadura Grown In Defined Media

This example illustrates that a defined medium supports Actinomadura growth and is
 25 more efficient for the bioconversion of compactin to pravastatin than complex medium. The
 chemically-defined medium, denoted Medium A, contains the following:

-16-

	<u>grams/liter</u>
sucrose	30
NaNO ₃	2
K ₂ HPO ₄	1
5 MgSO ₄ ·7H ₂ O	0.5
KCl	0.5
FeSO ₄	0.01
pH adjusted 7.2	

10 Actinomadura ATCC 55678 was grown for 45 hours in medium A and in complex YM medium (yeast extract 3g, malt extract 3g, peptone 5g, glucose 10g, per liter, pH 6.5), 5 ml/250 ml flask, at 28°C with shaking at 220 rpm. Compactin (250 µg/ml) was added at 45 hours. Incubation proceeded for 8 hours. The biomass was then measured, and it was about 25% less for cells grown in Medium A than for cells grown in YM medium. Despite fewer
15 cells, bioconversion of compactin to pravastatin was higher for cells grown in Medium A than for cells grown in YM medium. In YM there was 6.7% conversion; in Medium A there was 13% conversion.

Example 5: Enzymatic Conversion of Compactin to Pravastatin by

20 Cell Free Extracts Of Actinomadura Depends on NADPH

This example illustrates that cell free extracts of Actinomadura convert compactin to pravastatin, and that NADPH is a better H⁺ donor than NADH for this enzymatic conversion.

A cell free extract of Actinomadura was obtained by growing a 150 ml culture of Actinomadura ATCC 55678 for 2 days in YM medium as described in Example 1. The cells
25 were harvested by centrifugation at 15,000 rpm for 30 min. Cells were resuspended in 20 ml fresh YM medium. Na compactin was added to about 500 µg/ml and incubated at 29°C. 220 rpm for 3 hours. During this period, 196 µg/ml compactin disappeared and 70 µg/ml pravastatin appeared. Cells were harvested by centrifugation, washed twice with cold water and once with buffer A (80 mM Tris-HCl buffer, pH 7.4, supplemented with 20% glycerol and
30 2 mM DTT). The cells were resuspended in 5 ml buffer A and stored at -80°C. The cell suspension was later thawed and disrupted in a French Press. The homogenate was

centrifugated at 30,000 rpm for 60 min. The supernatant was stored at -80°C as cell free extract.

The reaction conditions were: 160 µl cell free extract, 0.26 mM NADH or NADPH, 0.23 mM compactin, 220 µl total volume, 30°C, 250 rpm. A control of buffer A without cell free extract was included. The amount of pravastatin was measured by HPLC as described in Example 1. The results are shown in Table 6.

Table 6. Comparison of NADH and NADPH as H⁺ Donors
in Conversion by Cell Free Extracts

of Actinomadura

	NADH		NADPH		control	
time (hours)	5	21	6	21	6	22
PV (µg/ml)	2.8	2.8	12.8	11.9	0	0

The results show that there is enzymatic conversion of compactin to pravastatin by cell free extracts and that it is better with NADPH as a H⁺ donor than with NADH.

Example 6: Enzymatic Conversion of Compactin to Pravastatin
from "Induced" and Uninduced Actinomadura Measured
in Cell Free Extracts

This example illustrates that, as measured in cell free extracts, the enzymatic conversion of compactin to pravastatin in Actinomadura does not require induction and that therefore the Actinomadura hydroxylase is a constitutive enzyme.

Cell free extracts were made both from compactin "induced" cells and cells without induction. Actinomadura ATCC 55678 cells were grown for 2 days as described in Example 2, except that the temperature was 29°C, harvested and divided into two flasks. Compactin was then added to one of these flasks at a concentration of about 300 µg/ml (the "induced" culture). Both flasks were incubated at 29°C, 250 rpm for 3 hours. Cells were harvested at this time and cell free extracts were made with a French Press as described in Example 4. The reaction conditions were: 160 µl cell free extract, 0.26 mM NADPH, 0.23 mM compactin.

220 µl total volume, 30°C, 250 rpm, incubated for 60 minutes. Enzyme conversion of the cell-free extract reaction was determined by HPLC as described in Example 1, at 0, 10, 30 and 60 minutes. The results are shown in Table 7.

Table 7: Conversion by "Induced" and Uninduced Cell
Free Extracts of Actinomadura

	Uninduced				"Induced"			
time (min)	0	10	30	60	0	10	30	60
PV (µg/ml)	0	0.98	6.7	12.7	0	2.0	5.0	9.1
CP (µg/ml)	97.2	77.6	69.5	68.5	90.2	80.6	67.2	56.1
specific rate (µg/min/g protein)	18				11			

These results indicate that "induction" did not increase the rate of enzymatic conversion. Therefore, the Actinomadura hydroxylase enzyme is a constitutive enzyme.

Example 7: Determination of Cytochrome P450 in Cell Free

Extracts of Actinomadura

This example illustrates that the hydroxylase activity of Actinomadura is not a cytochrome P450 enzyme. Cell free extracts of Actinomadura 55678 were prepared as described in Example 5. Preparations 1, 2 and 3 were resuspended in buffer A (80 mM Tris-HCl pH 7.4 supplemented with 2 mM DTT and 20% glycerol) and preparation 4 was resuspended in phosphate buffer. The reaction conditions were: 160 µl cell free extract, 0.26 mM NADPH, 0.23 mM compactin, 220 µl total volume, 30°C, 250 rpm. The hydroxylase activity was measured by HPLC as described in Example 1.

The cytochrome P450 activity was measured as follows. Two milligrams of sodium dithionate were added to 3 ml of cell free extract of Actinomadura ATCC 55678 to reduce P450. The cell free extract was put in a sample cell and a reference cell in a Beckman Model 24 spectrophotometer. The absorbance from 400 nm to 500 nm was recorded. The cell free extract in the sample cell was subjected to bubbling with CO for 1 min. to form a P450-CO

complex. The spectrum was recorded again. The difference between these two spectra is the P450 difference spectrum.

The P450 content and hydroxylase activity in the different cell free extract preparations are shown in Table 8. The different numbers for the cell free extract (CFE) preparations indicate independent cell free extract preparations.

Table 8. Hydroxylase and P450 Specific Activities
of Different Cell Free Extract Preparations
of Actinomadura

10

CFE Prep.	Buffer	Days of Cell Growth	"Induction"	Hydroxylase Activity (mg/min/g prot.)	P450 Peak Height (ABS./g prot.)
1	Buffer A	4	+	13	8
15 1	Buffer A	4	-	11	2
2	Buffer A	3	-	11	1
3	Buffer A	2	+	15	4
3	Buffer A	3	-	4.6	4
4	Phosphate	2.5	+	31	3
20 4	Phosphate	2.5	-	10	2

There was no correlation between the hydroxylase activity and the cytochrome P450 activity. Therefore, the hydroxylase activity derived from Actinomadura is not a cytochrome P450 enzyme.

Example 8: Effect of Various Factors on Conversion of Compactin to Pravastatin in Cell
Free Extracts of Actinomadura

This example illustrates that Mg^{++} , ATP and ascorbic acid, either alone or in combination, but not Fe^{++} or Fe^{+++} , stimulate conversion of compactin to pravastatin in Actinomadura cell free extracts. Mg^{++} is a common cofactor of many enzymes. ATP is a common energy provider for enzymatic reactions. Fe^{+++} and Fe^{++} are in the active site of

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many oxygenases. Ascorbic acid is a reducing agent often required for oxygenase reactions. The reaction conditions were 160 μ l cell free extract, 0.72 mM NADPH, 0.2 mM compactin, 211 μ l total volume, 30°C, 250 rpm, 60 min. The factors were added at time zero, at the following concentrations: 2.4 mM $MgCl_2$, 0.09 mM $FeSO_4$, 0.1 mM $FeCl_3$, 1.9 mM ATP and 12 mM ascorbic acid. The results are shown in Tables 9 and 10.

Table 9. Effects of $MgCl_2$, $FeSO_4$, $FeCl_3$, ATP and Ascorbic Acid on Cell Free Extracts Derived From Actinomadura

	no add.	$MgCl_2$	$FeSO_4$	$FeCl_3$	ATP+ascb
PV (μ g/ml)	3.2	4.4	2.3	3.3	11.3
CP (μ g/ml)	62.4	60.2	64.3	57.9	49.9
PV (μ g/min/g)	6.6	8.7	6.4	6.6	23

Table 10. Effects of $MgCl_2$, ATP and Ascorbic Acid on Cell Free Extracts Derived from Actinomadura

	CFE Uninduced				CFE "Induced"	
$MgCl_2$ (mM)	0	2.4	0	0	0	2.4
ATP (mM)	0	1.9	3.8	0	0	1.9
ascb (mM)	0	12	0	23	0	12
PV (μ g/ml)	2.9	11.1	7.5	8.3	2.8	7.8
CV (μ g/ml)	35.2	24.6	31.5	27.0	38.2	30.5
PV (μ g/min/g)	6.4	24	17	18	7.2	20

Example 9: Production of Pravastatin By Contacting a Pregrown Culture of Paecilomyces With a Starting Culture of Actinomadura

The fungus Paecilomyces sp. M2016 (Endo et al., J. Antibiotics 39:1609-1610 (1986)), is grown in a medium containing glucose 3.5%, starch 1%, soybean meal 2%, meat extract 0.5%, peptone 0.5%, NaCl 0.2%, KH_2PO_4 0.05%, and $MgSO_4 \cdot 7H_2O$ 0.05%, pH

adjusted to 5.8. Erlenmeyer flasks of 250 ml volume, each of which contains 20 ml of the medium, are sterilized at 121°C for 15 minutes. Each flask is inoculated with a platinum loop of *Paecilomyces* sp. M2016 from a slant culture, and is cultured with shaking at 250 rpm, 25°C for 7 days. The compactin produced is detected by HPLC as described in Example 1.

- 5 The produced compactin is 40 µg/ml. The pH is then adjusted to 7.2. A seed culture of *Actinomadura* ATCC 55678 is prepared as described in Example 2 and is added into the 7 day culture of *Paecilomyces* sp. After one day incubation at 28°C, 250 rpm, the pravastatin in the culture is detected by HPLC as described in Example 1. The concentration of pravastatin in the culture is 30 µg/ml.

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Example 10: Production of Pravastatin By Contacting
a Starting Culture of *Paecilomyces* With a
Cell Free Extract of *Actinomadura*

- A culture of the fungus *Paecilomyces* sp. M2016 is prepared as described in Example
15 9, except that the culture is mixed immediately after inoculating from the slant culture (without 7 days of pregrowth). A cell free extract of *Actinomadura* ATCC 55678 is prepared as described in Example 5. The culture of *Paecilomyces* sp. is adjusted to pH 7.5. 5 ml of the *Actinomadura* cell free extract is added to 20 ml of the *Paecilomyces* sp. culture. The mixture is incubated at 30°C, 250 rpm for 2 hours. The pravastatin produced from the compactin is
20 examined by HPLC as described in Example 1. The pravastatin concentration in the culture is 30 µg/ml.

Example 11: Treating High Blood Cholesterol Levels

- This example illustrates a method for lowering the cholesterol level in a human with
25 pravastatin derived from an *Actinomadura* hydroxylase that converts compactin to pravastatin. The patient is given 20 mg of this pravastatin orally, in the form of a pill, twice a day. Administration is carried out for a period of 3 months. This treatment results in a reduction of the patient's blood cholesterol level.

- Those skilled in the art will be able to ascertain, using no more than routine
30 experimentation, many equivalents of the specific embodiments of the invention described herein. These and all other equivalents are intended to be encompassed by the following claims.

CLAIMS

1. A method for converting compactin to pravastatin, comprising:
providing said compactin; and
contacting said compactin with an agent derived from Actinomadura under conditions
5 in which said agent converts compactin to pravastatin.
2. The method of claim 1 further comprising the step of isolating said pravastatin.
3. The method of claim 1 wherein said agent is a hydroxylation enzyme produced by said
10 Actinomadura.
4. The method of claim 3 wherein said hydroxylation enzyme is a hydroxylase that is
cytochrome P450 system-independent.
- 15 5. The method of claim 3 wherein said hydroxylation enzyme is constitutive.
6. The method of claim 1 wherein said compactin is provided by providing a
microorganism that produces said compactin.
- 20 7. The method of claim 6 wherein said microorganism is selected from the group
consisting of a fungus and a bacterium.
8. The method of claim 1 wherein said compactin is provided by providing a cell free
extract of a microorganism that produces said compactin.
- 25 9. The method of claim 1 wherein said compactin is provided by providing cell free
culture media from a pregrown culture of a microorganism that produces said compactin.
10. The method of claim 1 wherein said compactin is provided by providing a solution
30 comprising compactin.

11. The method of claim 1 wherein said compactin is provided by providing substantially purified compactin.

12. The method of claim 1, wherein said compactin is provided in shots.

13. The method of claim 1 wherein said compactin is contacted with said agent by contacting whole cells of said Actinomadura with said compactin.

14. The method of claim 1 wherein said compactin is contacted with said agent by contacting a cell free extract of said Actinomadura with said compactin.

15. The method of claim 1 wherein said compactin is contacted with said agent by contacting cell free culture media from a pregrown culture of said Actinomadura with said compactin.

16. The method of claim 1 wherein said compactin is contacted with said agent by contacting a solution comprising said agent of said Actinomadura with said compactin.

17. The method of claim 6 wherein said compactin is contacted with said agent by contacting a pregrown culture of said microorganism that produces said compactin with a pregrown culture of said Actinomadura.

18. The method of claim 6 wherein said compactin is contacted with said agent by contacting a pregrown culture of said microorganism that produces said compactin with a starting culture of said Actinomadura.

19. The method of claim 6 wherein said compactin is contacted with said agent by contacting a starting culture of said microorganism that produces said compactin with a pregrown culture of said Actinomadura.

20. The method of claim 6 wherein said compactin is contacted with said agent by contacting a starting culture of said microorganism that produces said compactin with a starting culture of said *Actinomadura*.
- 5 21. The method of claim 6 wherein said compactin is contacted with said agent by contacting a cell free extract of said microorganism that produces said compactin with a culture of said *Actinomadura*.
22. The method of claim 6 wherein said compactin is contacted with said agent by
10 contacting a culture of said microorganism that produces said compactin with a cell free extract of said *Actinomadura*.
23. The method of claim 6 wherein said compactin is contacted with said agent by contacting a cell free extract of said microorganism that produces said compactin with a cell
15 free extract of said *Actinomadura*.
24. The method of claim 1, wherein conversion of said compactin to said pravastatin is at least about 50%.
- 20 25. The method of claim 1, wherein conversion of said compactin to said pravastatin is at least about 80%.
26. A cell free extract derived from *Actinomadura*, comprising an agent that converts compactin to pravastatin.
- 25 27. The cell free extract of claim 26 wherein said cell free extract is derived from ruptured cells of said *Actinomadura* from which particulate cellular debris has been essentially removed.
- 30 28. The cell free extract of claim 26 wherein said agent is a hydroxylase.

29. The cell free extract of claim 28 wherein said hydroxylase is a constitutive enzyme, wherein the enzymatic activity of said hydroxylase is stimulated by any of ATP, ascorbic acid and Mg^{++} , but not stimulated by any of Fe^{++} and Fe^{+++} , and wherein said hydroxylase is cytochrome P450 system-independent.

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30. The cell free extract of claim 26 wherein conversion of said compactin to said pravastatin is at least about 50%.

31. The cell free extract of claim 26 wherein conversion of said compactin to said pravastatin is at least about 80%.

10

32. A hydroxylase from Actinomadura that converts compactin to pravastatin, said hydroxylase being a constitutive enzyme; the activity of said hydroxylase being stimulated by any of ATP, ascorbic acid and Mg^{++} , but not by Fe^{++} or Fe^{+++} ; and said hydroxylase being cytochrome P450 system-independent.

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33. Purified Actinomadura 55678, comprising an agent for converting compactin to pravastatin.

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34. The Actinomadura of claim 33 wherein said agent is a hydroxylase.

35. A method for treating a mammal to lower the blood cholesterol level of the mammal, comprising:

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providing pravastatin, said pravastatin being derived from compactin by contacting said compactin with an agent derived from Actinomadura that converts compactin to pravastatin; and administering said pravastatin to a mammal in need of such treatment to cause a lower blood cholesterol level in the mammal.

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36. The method of claim 35 wherein said agent is a hydroxylase.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09464

A. CLASSIFICATION OF SUBJECT MATTER IPC(6) : C12N 1/20, 9/14; C12P 1/04, 17/06 US CL : 435/125, 195, 252.1 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) U.S. : 435/125, 195, 252.1 Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US, A, 4,410,629 (TERAHARA ET AL) 18 October 1983, see entire disclosure.	1-5 8-16 24-32 35-36 6-7 17-23
Y		
<input type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
A	document defining the general state of the art which is not considered to be of particular relevance	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
E	earlier document published on or after the international filing date	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
L	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
O	document referring to an oral disclosure, use, exhibition or other means	*Z* document member of the same patent family
P	document published prior to the international filing date but later than the priority date claimed	
Date of the actual completion of the international search 01 AUGUST 1996		Date of mailing of the international search report 19 SEP 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230		Authorized officer HERBERT L. LILLING Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US96/09464

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims Nos.: 33-34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

the specification does not recite the properties of the microorganism other than the functional activity. A proper search and examination cannot be submitted based on the lack of data in the specification for the Actinomadura strain.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees